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REVIEW

Investigating cellular identity and manipulating cell fate using induced pluripotent stem cells

Tohru Sugawara[1], Koichiro Nishino[2], Akihiro Umezawa[1] and Hidenori Akutsu[1]*

Abstract

Induced pluripotent stem (iPS) cells, obtained from reprogramming somatic cells by ectopic expression of a defined set of transcription factors or chemicals, are expected to be used as differentiated cells for drug screening or evaluations of drug toxicity and cell replacement therapies. As pluripotent stem cells, iPS cells are similar to embryonic stem (ES) cells in morphology and marker expression. Several types of iPS cells have been generated using combinations of reprogramming molecules and/or small chemical compounds from different types of tissues. A comprehensive approach, such as global gene or microRNA expression analysis and whole genomic DNA methylation profiling, has demonstrated that iPS cells are similar to their embryonic counterparts. Considering the substantial variation among iPS cell lines reported to date, the safety and therapeutic implications of these differences should be thoroughly evaluated before they are used in cell therapies. Here, we review recent research defining the concept of standardization for iPS cells, their ability to differentiate and the identity of the differentiated cells.

The potential of stem cells and reprogramming

During mammalian development, cells in the developing fetus gradually become more committed to their specific lineage. The cellular differentiation process specializes to achieve a particular biological function in the adult, and the potential to differentiate is lost. Cellular differentiation has traditionally been thought of as a unidirectional process, during which a totipotent fertilized zygote becomes pluripotent, multipotent, and terminally differentiated, losing phenotypic plasticity (Figure 1). However, recent cloning experiments using nuclear transplantation have demonstrated that the epigenetic constraints imposed upon differentiation in mammalian oocytes can be released and the adult somatic nucleus restored to a totipotent embryonic state [1]. This process, a rewinding of the developmental clock, is termed nuclear reprogramming.

Embryonic stem (ES) cells derived from the inner cell mass of the mammalian blastocyst, an early-stage embryo, were first established from mice by Evans and Kaufman in 1981 [2]. Approximately two decades later, a human ES (hES) cell line was established by Thomson and colleagues [3]. ES cells possess a nearly unlimited capacity for self-renewal and pluripotency: the ability to differentiate into cells of three germ layers. This unique property might be useful to generate a sufficient amount of any differentiated cell type for drug screening or evaluations of drug toxicity and for cell replacement therapy. In addition, pluripotent stem cells provide us with an opportunity to understand early human embryonic development and cellular differentiation. Pluripotent ES cells are spun off directly from pre-implantation embryos [2-5]. To induce the somatic cell back to a pluripotent state, a strategy such as nuclear transplantation is fraught with technical complications and ethical issues. Thus, the direct generation of pluripotent cells without the use of embryonic material has been deemed a more suitable approach that lends itself well to mechanistic analysis and has fewer ethical implications [6].

In a breakthrough experiment, Takahashi and Yamanaka [7] identified reprogramming factors normally expressed in ES cells, Oct3/4, Sox2, c-Myc, and Klf4, that were sufficient to reprogram mouse fibroblasts to become pluripotent stem cells closely resembling ES cells. Because they were induced by the expression of defined factors, these cells were termed induced pluripotent stem (iPS) cells [7]. Since this landmark report in 2006, the technology has been rapidly confirmed among a number of species, including humans [8,9], rhesus monkeys [10], rats [11,12], rabbits [13], pigs [14] and two endangered primates [15]. In addition, mouse iPS (miPS) cells can be derived from various cell types, including fibroblasts [7,16], neural cells [17,18], liver cells [19], pancreatic β

*Correspondence: hakutsu@nch.go.jp
[1]Department of Reproductive Biology, Center for Regenerative Medicine, National Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan
Full list of author information is available at the end of the article

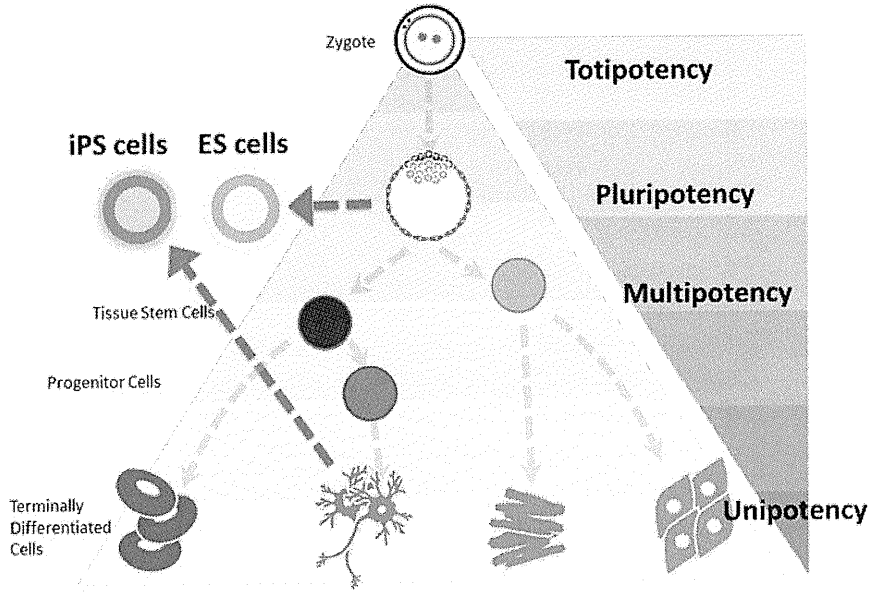

Figure 1. Hierarchical potential of stem cell development. A totipotent cell, such as a zygote and a blastomere of an early pre-implantation embryo, can give rise to all of the cell types in the whole body and the extraembryonic tissues. During mammalian development, pluripotent cells of the inner cell mass differentiate to give rise to lineage-committing stem cells and progenitor cells, and finally terminally differentiated cells by losing differential potential. Embryonic stem (ES) cells are spun off directly from the inner cell mass of blastocysts and induced pluripotent stem (iPS) cells are generated by reprogramming differentiated cells back to the pluripotent state. ES cells and iPS cells seem to have highly similar pluripotential properties.

cells [20], and terminally differentiated lymphocytes [21,22]. Subsequently, human iPS (hiPS) cells have been derived from various readily accessible cell types, including skin fibroblasts [8,9], keratinocytes [23], gingival fibroblasts [24], peripheral blood cells [25,26], cord blood cells [27,28] and hair follicle cells [29].

These products and systems for this state-of-the art technology provide useful platforms for disease modeling and drug discovery, and could enable autologous cell transplantation in the future. Given the methodologies for studying disease mechanisms, disease- and patient-specific iPS cells can be derived from patients. For applying novel reprogramming technologies to biomedical fields, we need to determine the essential features of iPS cells. In this review, we summarize the functional and molecular properties of iPS cells in comparison to ES cells in the undifferentiated state and with regard to differentiation efficiency. We also review evaluation for the types of differentiated cells derived from of iPS and ES cells and compare the functions of these.

Reprogramming methods and factors

Although the establishment of iPS cells from somatic cells is technically easier and simpler compared with nuclear transplantation, several variables should be considered due to variations in the reprogramming process, including the reprogramming factors used, the combinations of factors and the types of donor-parent cells. Each method has advantages and disadvantages, such as efficiency of reprogramming, safety, and complexity, with the process used affecting the quality of the resultant iPS cells. Initial generations of miPS and hiPS cells employed retroviral and lentiviral vectors [7-9] (Table 1), carrying the risk of both insertional mutagenesis and oncogenesis due to misexpression of the exogenous reprogramming factors, Oct3/4, Sox2, c-Myc, and Klf4. In particular, reactivation of c-Myc increases tumorigenicity in the chimeras and progeny mice, hindering clinical applications.

Since the initial report of iPS cell generation, modifications to the reprogramming process have been made in order to decrease the risk of tumorigenicity and increase reprogramming efficiency [30-32]. Several small molecules and additional factors have been reported to enhance the reprogramming process and/or functionally replace the role of some of the transcription factors (Table 1). Small molecules are easy to use and do not result in permanent genome modifications, although iPS generation using only a set of small molecules has not been reported. Combining small molecule compounds with reprogramming factors would enhance reprogramming efficiency. Integration-free hiPS cells have been established using Sendai virus [33,34], episomal plasmid vectors [35,36], minicircle vectors [37], and direct protein

Table 1. Various methods used for reprogramming

Method	Factors[a]	Sources	Enhancement factors
Adenovirus	OSKM	Mouse fibroblast and liver cells [77], human embryonic fibroblast cells [78]	
Bacteriophage	OSKM	Mouse embryonic fibroblasts, human amniocytes [79]	
Episomal vector	OSKMNL	Human foreskin fibroblasts [36]	SV40LT
		Human fibroblasts, adipose stem cells, cod blood cells [80]	SV40LT, LIF, MEK/GSK3b/TGFBR inhibitor, HA-100/human
	OSKM*L	Human dermal fibroblasts [81]	p53 shRNA
Lentivirus	OSKM	Mouse pancreatic b cells [20]	
		Human adult fibroblasts [82]	p53 siRNA, UTF1
		Mouse B lymphocytes [21]	C/EBPa or Pax5 shRNA
	OSNL	Human newborn foreskin [9]	
		Human fibroblasts [83]	SV40LT
	OSKMNL	Human fibroblasts [84]	
	OSN	Gut mesentery-derived cells [85], human amnion-derived cells [86]	
	O	Human epidermal keratinocytes [87]	TGFBR/MEK1 inhibitor, PDK1 activator, sodium butyrate
Minicircle vector	OSNL	Human adipose stromal cells [37]	
microRNA	miR-200c, 302a/b/c/d, 369-3p/5p	Human and mouse adipose stromal cells [64]	
mRNA	OSNL	Human fibroblasts [88]	
	OSKM(L)	Primary human neonatal epidermal keratinocytes [40]	
piggyBAC	OSKM	Human and mouse embryonic fibroblasts [89,90]	
Plasmid	OSKM	Mouse embryonic fibroblasts [35,91]	
	OSNL	Human foreskin fibroblasts [92]	MEK inhibitor
Protein	OSKM	Mouse embryonic fibroblasts [38]	VPA
	OSKM	Human fibroblasts [39]	
Retrovirus	OSKM	Human fibroblasts [8], mouse fibroblasts [7], human keratinocytes [23], human peripheral blood cells [25]	
		Human fibroblasts, adipose stem cells [93]	Vitamin C, VPA
	OSK	Adult human dermal fibroblasts [30]	
		Mouse embryonic fibroblasts [94]	Wnt3a
		Rat liver progenitor cells [11]	MEK/ALK5/GSK3b inhibitor
		Mouse embryonic fibroblasts [93]	Vitamin C
		Mouse and human fibroblasts [32]	GLIS1
		Mouse embryonic fibroblasts [95]	mmu-miR-106a/18b/20b/19b/92a/363 or 302a/302b/302c/302d/367
		Human fibroblasts [96]	hsa-miR-302b or 372
	OK	Mouse embryonic fibroblasts [97]	BIX01294, BayK8644
		Neonatal human epidermal keratinocytes [98]	GSK3b inhibitor
	O	Mouse neural stem cells [99]	
		Mouse fibroblasts [100]	GSK3b inhibitor, vitamin C, BMP4
	hsa-miR-302a/b/c/d	Human skin cancer cells [101]	
Sendai virus	OSKM	Human fibroblasts [33], human cord blood [102]	

[a]O, OCT3/4; S, SOX2; K, KLF4; M, C-MYC; M*, L-MYC; N, NANOG; L, LIN28. ALK, anaplastic lymphoma kinase; BayK8644, L-type calcium channel agonist; BIX01294, histone methyltransferase inhibitor; BMP, bone morphogenetic protein; GSK, glycogen synthase kinase; GLIS, GLI (MIM 165220)-related Kruppel-like zinc finger; LIF, leukemia inhibitory factor; PDK, pyruvate dehydrogenase kinase; shRNA, short hairpin RNA; siRNA, small interfering RNA; TGFBR, transforming growth factor beta receptor; UTF, undifferentiated transcription factor; VPA, valproic acid (histone deacetylase inhibitor).

[38,39] or mRNA [40] delivery (Table 1). However, direct delivery of proteins or RNA requires multiple transfection steps with reprogramming factors compared to other viral integration methods.

iPS cells appear indistinguishable from ES cells

The key to generating iPS cells is to revert somatic cells to a pluripotent state that is molecularly and functionally equivalent to ES cells derived from blastocysts (Table 2). Reprogrammed iPS cells express endogenous transcription factors that are required for self-renewal and maintenance of pluripotency, such as OCT3/4, SOX2, and NANOG, and for unlimited proliferation potential, such as TERT [8,9]. Telomeres were elongated in iPS cells compared to the parental differentiated cells in both humans and mice [41,42]. In addition, cellular organelles such as mitochondria within hiPS cells were morphologically and functionally similar to those within ES cells [43]. The establishment of an ES cell-like epigenetic state is a critical step during the reprogramming of somatic cells to iPS cells and occurs through activation of endogenous pluripotency related genes. Bisulfite genomic sequencing has shown that the promoter regions of the pluripotency markers NANOG and OCT3/4 are significantly demethylated in both hiPS and hES cells [8,44], and the heterogeneity of X chromosome inactivation in hiPS cells is similar to that in ES cells [45].

In terms of multilineage differentiation capacity, miPS cells from various tissue types have been shown to be competent for germline chimeras [19,32,46]. It was shown that miPS cells generated viable mice via tetraploid complementation [47,48]. In the mouse system, iPS cells retain a developmental pluripotency highly similar to that of mouse ES cells according to the most stringent tests. Although it has been generally assumed that autologous cells should be immune-tolerated by the recipient from whom the iPS cells were derived, Zhao and colleagues [49] reported that the transplantation of immature miPS cells induced a T-cell-dependent immune response even in a syngeneic mouse. This is an unexpected result but some issues need to be considered: the influence of the cell type of origin on the immunogenic properties of resultant iPS cells must be explored; undifferentiated iPSCs should never be used for medical applications; and the mechanism of aberrant gene expression should be determined [50].

To functionally assay hiPS cells, teratoma formation and histological analysis to confirm the presence of structures derived from all three germ layers are currently regarded as the most rigorous ways to prove pluripotency of human stem cells. Recently, Müller and colleagues [51] proposed the use of PluriTest, a bioinformatics assay for the prediction of stem cell pluripotency using microarray data. Such microarray-based gene expression and DNA methylation assays are low cost, save time and have been used to evaluate the differentiation efficiency of individual cell lines [52].

ES and iPS cells differ in their epigenetic signatures

Epigenetic modification of the genome ensures proper gene activation for maintaining the pluripotency of stem cells and also differentiation into proper functional cells [1]. It will be important to assess the epigenetic state of hiPS cells compared to donor parent cells and embryo-derived hES cells. Analyzing epigenetic states, such as histone modifications and DNA methylation of selected key pluripotency genes, showed the chromatin state of iPS cells to be identical to that of ES cells upon reprogramming (reviewed in [53]).

Genome-wide analyses of histone methylation patterns have demonstrated that iPS cells were clearly distinguished from their origin and similar to ES cells in the mouse [54]. All of these analyses, however, reported some differentially methylated regions (DMRs) between ES and iPS cells. Recent studies found that miPS cell lines retained the residual signatures of DNA methylation of the parental cells [55,56]. Additionally, some of the hyper-methylated regions in hiPS cells are also hyper-methylated in the original cells, meaning that an epigenetic memory is inherited during the reprogramming process through early passaging [57]. Parental cell-related DMRs and incomplete promoter DNA methylation contributed to aberrant gene expression profiles in iPS cells to some extent [58]. The other remaining DMRs appeared to be aberrantly methylated regions established in iPS cells during reprogramming that differ from both the parental cells and the ES cells. Nishino and colleagues [57] compared methylation profiles of six hiPS cell lines and two hES cell lines and reported that approximately 60% of DMRs were inherited and 40% were iPS-specific. Interestingly, most aberrant DMRs were hyper-methylated in iPS cell lines [57,59]. Lister and colleagues [60] also compared methylation profiles in five hiPS cell lines and two hES cell lines and found that the hiPS cells shared megabase-scale DMRs proximal to centromeres and telomeres that display incomplete reprogramming of non-CpG methylation, and differences in CpG methylation and histone modifications in over a thousand DMRs between hES and hiPS cells. Although lots of studies have detected several DMRs shared between iPS and ES cells, no DMRs were found in all iPS cell lines.

microRNAs (miRNAs), which are also epigenetically regulated, play critical roles in gene regulation by targeting specific mRNAs for degradation or by suppressing their translation. Several studies recently reported the presence of unique clusters of miRNAs, such as the human and mouse miR-302 cluster in ES and iPS cells [61,62]. These miRNAs enhance the transcription factor-mediated

Table 2. Characteristics of human induced pluripotent stem cells compared to human embryonic stem cells

Variable factor	Characteristics	Characteristics of hiPS cells
Cell source		Without the use of embryonic material Enable autologous cell transplantation
Technique for the generation of iPS cells		Simply trans-activating several transcription factors and/or exposure to several chemical components Variables due to reprogramming methods and/or donor-parental cells
Morphology		Flat and tightly packed colony identical to hES cells
Proliferation potency		Unlimited self-renewal identical to hES cells
Pluripotency	Genes	OCT3/4, NANOG, SOX2 expression identical to hES cells
	Gene promoter	OCT3/4, NANOG demethylation identical to hES cells
	Cell surface antigens	SSEA3, SSEA4, TRA-1-60, TRA-1-81 positive identical to hES cells
	Teratoma formation	Differentiation into three germ layers similar to hES cells
X chromosome inactivation (XCI)		Heterogeneity (complete XCI, partial XCI, pre-XCI) similar to hES cells
Mitochondria	Genome	Accumulated mtDNA mutations transmitted from parental cells Genetic mutations during reprogramming
	Morphology	Globular shape with only small christae similar to hES cells and ES cell-like distribution
	Function	Expression of nuclear factors involved in mitochondrial biogenesis
Telomere		Telomere elongation and ES cell-like telomerase activity
Epigenetic profile		Retention of somatic memory and aberrant methylation during the reprogramming process
microRNAs		Up-regulation of miR-302 cluster identical to hES cells

ES, embryonic stem; hES, human embryonic stem; hiPS, human induced pluripotent stem; iPS, induced pluripotent stem; mtDNA, mitochondrial DNA; XCI, X chromosome inactivation.

reprogramming process (Table 1). Furthermore, two independent groups generated human and mouse iPS cells by adding only miRNAs in the absence of any additional protein factors [63,64]. Two reports have described a small number of differences in miRNA expression patterns between hiPS and hES cells [62,65], although our preliminary analysis showed that miR-372 and miR-373 are expressed at similar levels in both hiPS and hES cells and they were not detected in parental cells.

Changes of epigenetic profiles in iPS cells during culture

It is possible that iPS cells vary in their epigenetic profiles and degree of pluripotency due to differential levels of reprogramming. Nishino and colleagues [66] investigated the effect of continuous passaging on DNA methylation profiles of seven hiPS cell lines derived from five cell types. Although *de novo* DMRs that differ between hES and hiPS cells appeared at each passage, their number decreased and they disappeared with passaging; therefore, the total number of DMRs that differ between ES and iPS cells decreased with passaging. Thus, continuous passaging of the iPS cells diminished the epigenetic differences between iPS and ES cells, implying that iPS cells lose the characteristics inherited from the parental cells and develop to very closely resemble ES cells over time [66]. They also confirmed that the transgenes were silenced at each passage examined, indicating that the number of DMRs that differed between ES and iPS cells decreased during the transgene-independent phase. This is consistent with a study by Chin and colleagues [67], who found that the gene expression profile of hiPS cells appeared to become more similar to that of hES cells upon extended passaging. Although comprehensive DNA methylation profiles have recently been generated for hiPS cells, it seems harder to determine common DMR sites during iPS reprogramming. There are three possible explanations for the many inconsistent results regarding iPS cell-specific DMRs: hiPS cells have only been analyzed at a single point of passage in almost all studies; inherited methylation from parental cells is non-synchronous and stochastic, much like aberrant methylation, rather than deterministic [66]; and the aberrant hypermethylation at DMRs in iPS cells occurs 'stochastically' throughout the genome during passaging [66].

Genetic changes during reprogramming and extended culture

Genomic stability is critical for the clinical use of hiPS cells. The occurrence of genetic changes in hES cells is now well known as well as that the karyotypic changes observed are nonrandom and commonly affect only a few chromosomes [68]. Recent studies revealed that the

reprogramming process and subsequent culture of iPS cells *in vitro* can induce genetic changes. Three types of genomic abnormalities were seen: aberrations of somatic cell origin, aberrations present in early passages but not of apparent somatic cell origin, and aberrations acquired during passaging. Notably, the high incidence of chromosome 12 duplications observed by Mayshar and colleagues [69] caused significant enrichment for cell cycle-related genes, such as *NANOG* and *GDF3*. Another study reported that regions close to pluripotency-associated genes were duplicated in multiple samples [70]. Selection during hiPS cell reprogramming, colony picking and subsequent culturing may be factors contributing to the accumulation of mutations.

Impact of epigenetic differences on pluripotency

One of the goals of using hiPS cells is to generate functional target cells for medical screening and therapeutic applications. For these applications, it must be evaluated thoroughly whether small DMRs among ES and iPS cells affect the competency, differentiation propensities, stability and safety of iPS cells. It remains to be elucidated how the degree of these differences contributes to the variance in pluripotency among ES and iPS cells. Analysis of iPS cells obtained from mouse fibroblasts and hematopoietic and myogenic cells demonstrated that cellular origin influences the potential of miPS cells to differentiate into embryoid bodies and different cell types *in vitro*. In a related study, Kim and colleagues [56] compared the ability to differentiate to blood lineages of iPS cells derived from fibroblasts, neural cells, hematopoietic cells and ES cells in the mouse system, and demonstrated consistent differences in blood-forming ability - that is, blood derivatives showed more robust hematopoiesis *in vitro* than neural derivatives. Therefore, low-passage iPS cells derived from different tissues harbor residual DNA methylation signatures characteristic of their somatic tissue of origin, which favors their differentiation along lineages related to the parental cell, while restricting alternative cell fates. Similarly, Miura and colleagues [71] demonstrated that differences in gene expression in miPS cells derived from different types of parental cells result in variations in teratoma formation. These studies demonstrate that reprogramming to generate iPS cells is a gradual process that modifies epigenetic profiles beyond the acquisition of a pluripotent state.

Prediction for pluripotency and differentiation preference

Significant variation has been also observed in the differentiation efficiency of various hES cell lines [72]. Incomplete DNA methylation of somatic cells regulates the efficiency of hiPS cell generation [58], and selection of parental cell types influences the propensity for differentiation [73,74]. Such differences must be better understood before hES and hiPS cell lines can be confidently used for translational research. To predict a cell line's propensity to differentiate into the three germ layers, Bock and colleagues [52] performed DNA methylation mapping by genome-scale bisulfite sequencing and gene expression profiling using microarrays and quantified the propensity to form multiple lineages by utilizing a non-directed embryoid bodies formation assay and high-throughput transcript counting of 500 lineage marker genes in embryoid bodies using 20 hES cells lines and 12 hiPS cell lines over passages 15 to 30. They bioinformatically integrated these genomic assays into a scorecard that measures the quality and utility of any human pluripotent cell line. The resulting lineage scorecard pinpoints quantitative differences among cell-line-specific differentiation propensities. For example, one hES cell line that received a high score for endoderm differentiation performed well in directed endoderm differentiation, and other hES cell lines that received high scores for neural lineage differentiation efficiently differentiated into motor neurons. In addition, two hiPS lines that the scorecard predicted to have a low propensity to differentiate into the neural lineage were impaired in motor neuron-directed differentiation. On the other hand, other hiPS lines that the scorecard predicted to have a high propensity to differentiate into ectodermal and neural lineages were found to differentiate well into motor neurons. Therefore, the scorecard can detect lineage-specific differences in the differentiation propensities of a given cell line [52].

Functional assay for differentiated cells from iPS and ES cells

Although the propensity for differentiation could be predicted, it remains to be elucidated whether iPS cell-derived cells are functionally and molecularly the same as ES cell-derived cells. To address this issue, two studies conducted functional assays comparing differentiated neural cells derived from iPS cells to those derived from ES cells by marker gene expression and action potential measurements [75,76]. There was some variation in efficiency and quantitative differences in motor neuron generation among the lines, but the treatment of neuroepithelial cells from pluripotent stem cells with retinoic acid and sonic hedgehog resulted in the generation of iPS and ES cell lines with a neuronal morphology that expressed TUJ1. In addition, electrophysiological recordings using whole-cell patch clamping showed inward and outward currents, and it was concluded that ES cell- and iPS cell-derived neurons are similarly functional at a physiological level. These studies demonstrated that the temporal course and gene-expression pattern during

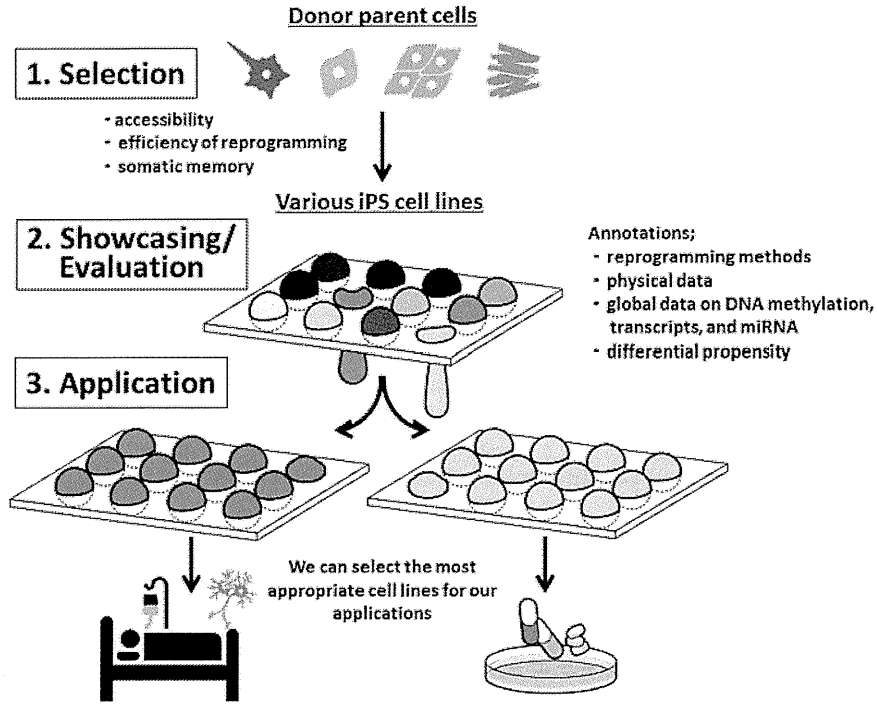

Figure 2. Workflow for human iPS cell applications. 1. Selection: choosing donor parent tissue considering accessibility, efficiency of reprogramming, and differential propensity. It would be useful to evaluate the expression of somatic memory genes, such as *C9orf64*, which reduces the efficiency of induced pluripotent stem (iPS) cell generation [58]. 2. Showcasing/evaluation: provides annotated information on reprogramming methods, culture conditions, physical data on stem cells, and global data on DNA methylation, transcription and microRNAs (miRNAs). It is very informative to integrate the genetic and epigenetic and biological data, such as differential propensity [52,76]. 3. Application: using annotation data, we can select the most appropriate iPS cell lines for our applications. Various hiPS cell lines (shown as differently shaded spheres) would be listed before further processing of the application. Valid cell lines (colored purple and blue) could be functionally and molecularly selected for appropriate applications, such as cell replacement therapy and/or drug screening.

neuroepithelial cell differentiation and production of functional neurons were nearly identical between ES and iPS cells, regardless of the reprogramming method, cellular origin, and differences between iPS and ES cells. These findings raise hopes of applying human iPS cells to the modeling of diseases and potential autologous cell transplantation.

It is important to acquire scientific information on pluripotential stem cells for further applications, such as industrial and clinical uses. Pluripotent stem cells, including disease-specific stem cells, could be showcased with useful annotation data and the most appropriate cell lines could be selected (Figure 2).

Conclusion

Many issues have yet to be resolved before the results of stem cell research can benefit the public in the form of medical treatments. In this review, we have discussed the substantial variation observed among pluripotent stem cells, including transcriptional and epigenetic profiles in the undifferentiated state, the ability to differentiate into various types of cells, and the functional and molecular nature of embryoid body or stem cell-derived differentiated cells. These results suggest that most, but not all, iPS cell lines are indistinguishable from ES cell lines, even though there is a difference between the average ES cell and the average iPS cell. Thus, ES and iPS cells should not be regarded as one or two well-defined points in the cellular space but rather as two partially overlapping point clouds with inherent variability among both ES and iPS cell lines [52,76]. Notably, human iPS cells seemed to be more variable than human ES cells. No single stem cell line may be equally powerful for deriving all cell types *in vitro*, implying that researchers would benefit from identifying the best cell lines for each application. Furthermore, for clinical use in the future, it is important to use both ES and iPS cells in research, and to standardize reprogramming methods, culture equipment and techniques and to optimize differentiation methods and evaluate the functions and tumorigenicity of differentiated cells.

This article is part of a review series on *Induced pluripotent stem cells*. Other articles in the series can be found online at http://stemcellres.com/series/ipsc

Abbreviations
DMR, differentially methylated region; ES, embryonic stem; hES, human embryonic stem; hiPS, human induced pluripotent stem; iPS, induced pluripotent stem; miPS, mouse induced pluripotent stem; miRNA, microRNA.

Competing interests
The authors declare that they have no competing interests.

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Author details
[1]Department of Reproductive Biology, Center for Regenerative Medicine, National Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan. [2]Laboratory of Veterinary Biochemistry and Molecular Biology, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen-Kibanadai-Nishi, Miyazaki, 889-2192, Japan.

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Feeder-Free and Serum-Free Production of Hepatocytes, Cholangiocytes, and Their Proliferating Progenitors from Human Pluripotent Stem Cells: Application to Liver-Specific Functional and Cytotoxic Assays

Naoko Nakamura,[1] Kumiko Saeki,[1] Masami Mitsumoto,[1] Satoko Matsuyama,[1] Miwako Nishio,[1] Koichi Saeki,[2] Mamoru Hasegawa,[2] Yoshiyuki Miyagawa,[3] Hajime Ohkita,[3] Nobutaka Kiyokawa,[3] Masashi Toyoda,[3] Hidenori Akutsu,[3] Akihiro Umezawa,[3] and Akira Yuo[1]

Abstract

We have established a serum- and feeder-free culture system for the efficient differentiation of multifunctional hepatocytes from human embryonic stem (ES) cells and three entirely different induced pluripotent stem (iPS) cells (including vector/transgene-free iPS cells generated using Sendai virus vector) without cell sorting and gene manipulation. The differentiation-inducing protocol consisted of a first stage; endoderm induction, second stage; hepatic initiation, and third stage; hepatic maturation. At the end of differentiation culture, hepatocytes induced from human pluripotent stem cells expressed hepatocyte-specific proteins, such as α-fetoprotein, albumin, α1 antitrypsin and cytochrome P450 (CYP3A4), at similar or higher levels compared with three control human hepatocyte or hepatic cell lines. These human iPS/ES cell-derived hepatocytes also showed mature hepatocyte functions: indocyanine green dye uptake (~30%), storage of glycogen (>80%) and metabolic activity of CYP3A4. Furthermore, they produced a highly sensitive hepatotoxicity assay system for D-galactosamine as determined by the extracellular release of hepatocyte-specific enzymes. Hepatoprotective prostaglandin E1 attenuated this toxicity. Interestingly, bile duct-specific enzymes were also detected after drug treatment, suggesting the presence of bile-duct epithelial cells (cholangiocytes) in our culture system. Electron microscopic studies confirmed the existence of cholangiocytes, and an immunostaining study proved the presence of bipotential hepatoblasts with high potential for proliferation. Differentiated cells were transferrable onto new dishes, on which small-sized proliferating cells with hepatocyte markers emerged and expanded. Thus, our differentiation culture system provides mature functional hepatocytes, cholangiocytes, and their progenitors with proliferative potential from a wide variety of human pluripotent stem cells.

Introduction

THE LIVER PLAYS CRITICAL ROLES for regulating metabolic homeostasis, because it is responsible for the metabolism, synthesis, and storage of nutrients. It is also well known that the liver is a central organ for the detoxification of drug compounds and other toxic substances taken into the human body. Thus, dysfunction of liver results in serious conditions, and liver/hepatocyte transplantation is the major therapeutic option for patients with chronic end-stage liver disease (Miro et al., 2006). However, the major limitation of cell-based therapies for liver disease is the availability of human hepatocytes.The use of embryonic stem (ES) cells may be the most effective strategy to develop hepatocytes that may be valuable in regenerative medicine and for pharmacological studies. Human ES cells proliferate infinitely *in vitro* while maintaining their potential to differentiate into almost all cell types (Thompson et al., 1998), and thus provide a potential source for obtaining hepatocytes. Several studies have demonstrated the capacity of human ES cells to differentiate into

[1]Department of Disease Control, Research Institute, National Center for Global Health and Medicine, Tokyo, Japan.
[2]DNAVEC Corporation, Ibaraki, Japan.
[3]Department of Reproductive Biology, Research Institute, National Center for Child Health and Development, Tokyo, Japan.

hepatocyte-like cells (Agarwal et al., 2008; Cai et al., 2007; Chiao et al., 2008; Duan et al., 2007, 2010; Hay et al., 2008a, 2008b; Ishii et al., 2008; Mfopou et al., 2010; Sasaki et al., 2009; Touboul et al., 2010; Zhao et al., 2009). However, most reports performed limited phenotypic and functional tests on the differentiated cells, and most differentiation culture methods include steps using animal-derived components unsuitable for clinical application. In addition, detailed studies on individual diversity of metabolism and toxicity for drugs, leading to tailor-made medicine, cannot be performed readily using only limited numbers of human ES cell lines.

Induced pluripotent stem (iPS) cells were generated directly from somatic cells as a result of the introduction of four reprogramming factors, Oct4, Sox2, Klf4, and c-Myc (Park et al., 2008; Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007), and shared many characteristics with ES cells, including multilineage differentiation potential, and intensive proliferation *in vitro*. In addition, the establishment of human iPS cells is ethically acceptable and does not require human oocytes. Thus, we may be able to obtain patient-specific functional cells for research into diseases, apply these cells to the regenerative medicine for therapeutic use, and use these cells for *in vitro* testing to satisfy industrial requirements, including drug discovery. However, as is the cases with human ES cells, it is not easy to regulate the differentiation of human iPS cells toward endoderm cells such as hepatocytes (Inamura et al., 2011; Liu et al., 2010; Si-Tayeb et al., 2010; Song et al., 2009; Sullivan et al., 2010). In addition, unlike human ES cells, most of human iPS cells have been generated via retrovirus/lentivirus vector systems, resulting in genomic integration of viral components into iPS cells (Park et al., 2008; Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007).

Here we report the establishment of a serum- and feeder-free method for hepatocyte differentiation of human iPS/ES cells (including virus-free iPS cells established using Sendai virus vector) (Fusaki et al., 2009), providing excellent tools for the evaluation of drug metabolism and hepatotoxicity. We succeeded in producing cholangiocytes and bipotential hepatoblasts and developed minimally invasive subculture methods of proliferating hepatocyte stem/progenitor cells under feeder-free conditions without using cell-sorting techniques. Our system will contribute to drug discovery and tailor-made medicine with the aim of dispensing the safest drugs for each individual.

Materials and Methods

Generation and culture of human iPS cells

A human iPS cell line, 253G1, was established by transducing human adult skin fibroblasts with retrovirus containing Oct3/4, Sox2, Klf4, and/or c-Myc, as described previously (Takahashi et al., 2007). Human iPS cell line #40 was generated from human fetal lung fibroblasts (MRC-5 cells), via procedures described by Yamanaka and colleagues (Takahashi et al., 2007) with slight modifications (Nagata et al., 2009; Toyoda et al., 2011).

We also established human iPS cell line SeV5 without integration of viral vector components from human neonatal fibroblasts using Sendai virus (SeV) vectors, as described previously (Ban et al., 2011; Fusaki et al., 2009). Human fibroblast cell line BJ from neonatal foreskin (ATCC, Manassas, VA) were infected with SeV vectors containing Oct3/4, Sox2, Klf4, or c-Myc and were then incubated for 6 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (PAA Laboratories GmbH, Linz, Austria). Then the cells were cultured on dishes coated with γ-irradiated murine embryonic fibroblasts (MEFs) in Primate ES cell medium (ReproCELL Inc., Tokyo, Japan) for 3 weeks. Human ES cell-like colonies were picked up using a micropipette and were further cultured on dishes coated with γ-irradiated MEFs. These human ES-like cells expressed several multipotent markers such as SSEA4, Oct3/4, and Nanog. SeV and transgenes in human iPS cells were diluted to undetectable levels during repeated passage for approximately 2 months and/or were deleted by high-temperature cultivation (at 39°C) for 7 days (Ban et al., 2011).

All human iPS cells were maintained on dishes coated with γ-irradiated MEFs as described previously (Gokoh et al., 2011).

Culture of human ES cells and normal human hepatocyte cell lines

The use of human ES cells was performed in accordance with the Guidelines on the Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science, and Technology of Japan, after approval by the institutional review board of the National Center for Global Health and Medicine. Human ES cells (KhES-1) (Suemori et al., 2006), provided by Kyoto University (Kyoto, Japan), were maintained as described previously (Nakahara et al., 2009a, 2009b; Saeki et al., 2009).

We used two human hepatic cell lines as positive control. HepG2 cell was purchased from Research Resources Bank of Japan Health Science Foundation (Osaka, Japan) and cultured in DMEM supplemented with 10% heat-inactivated FBS (PAA Laboratories). HepaRG cells (Gripon et al., 2002) was purchased from BIOPREDIC INTERNATIONAL (Rennes, France) and cultured on collagen IV-coated dish in General Purpose medium 670, Maintenance and Metabolism medium 620, or Induction medium 640 (BIOPREDIC INTERNATIONAL), according to the protocol of the supplier.

Hepatocyte differentiation of human iPS and ES cells in nonfeeder and serum-free culture

Before the induction of differentiation, human iPS and ES cells maintained on MEF were detached with dissociation liquid containing 0.25% trypsin (Invitrogen Corp., Carlsbad, CA), 1 mg/mL collagenase IV (WAKO Pure Chemical Industries, Osaka, Japan), 20% Knockout™ Serum Replacement (KSR) (Invitrogen), and 1 mM $CaCl_2$. Detached cells were collected into conical tube, and were then allowed to stand at room temperature to sediment iPS/ES cells. After appropriate periods (approximately a few minutes), MEF in the supernatant of the tube were aspirated and iPS/ES cells at the bottom of the tube were collected. Collected human iPS/ES cells were then cultured for 2–3 days on matrigel-coated dish in DMEM/F12 medium supplemented with 20% KSR (Invitrogen), 1% nonessential amino acid solution (Invitrogen), 1 mM sodium pyruvate solution, 100 μM 2-mercaptethanol, 2 mM L-glutamine, 20 U/mL penicillin, and 20 μg/mL streptomycin.

The protocol for differentiation induction culture is summarized in Figure 1. Human iPS/ES cells were culture on matrigel-coated dishes in RPMI1640 medium supplemented with 2 mM L-glutamine, 100 μM 2-mercaptoethanol, 20 U/mL penicillin 20 μg/mL streptomycin, and 0.29% recombinant human albumin (Mitsubishi Tanabe Pharma Corp., Osaka, Japan) in the presence of 100 ng/mL Activin A (PeproTech, Rocky Hill, NJ), and 25 ng/mL Wnt 3A (R&D Systems Inc., Minneapolis, MN) for 24 h, followed by another 24-h culture in the presence of Activin A alone in the presence of 0.2% KSR (Invitrogen). After 48 h from the beginning of differentiation culture, the cells were subjected to the next stage of culture and were cultured with mixtures of three cytokines, fibroblast growth factor-2 (FGF-2; PeproTech; 10 ng/mL), bone morphogenic protein-4 (BMP-4; Wako Pure Chemical Industry; 20 ng/mL) and Sonic hedgehog (Shh; R&D; 200 ng/mL) in the presence of 2% KSR for 5 days. Then, the cells were culture with hepatocyte growth factor (HGF; R&D; 20 ng/mL), FGF-2 (10 ng/mL), and BMP-4 (20 ng/mL) for another 5 days in similar medium. Finally, cells were culture in the presence of oncostatin M (R&D; 10 ng/mL) and dexamethasone (0.1 μM) for final hepatic maturation for 6 to 16 days.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cultured cells using TRIzol Reagent (Life Technologies co., Carlsbad, CA). For reverse transcription reactions, 1 μg RNA was reverse-transcribed using Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). A total of 0.5 μL of cDNA was used for PCR analysis. PCR amplification of different genes was performed using rTaq (Takara, Tokyo, Japan), with a program comprising 94°C for 5 min, 35 cycles of 94°C for 30 sec, 50–60°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 10 min. The sequence of the primers used are as follows: α-fetoprotein (AFP); a forward primer 5′-TTTTG GGACCCGAACTTTCC-3′ and a reverse primer 5′-CTCCT GGTATCCTTTAGCAACTCT-3′, albumin (ALB); a forward primer 5′-GGTGTTGATTGCCTTTGCTC-3′ and a reverse primer 5′-CCCTTCATCCCGAAGTTCAT-3′, α1-antitrypsin (AAT); a forward primer 5′-ACATTTACCCAAACTGTCCA TT-3′ and a reverse primer 5′-GCTTCAGTCCCTTTCTC GTC-3′, cytochrome P450 3A4 (CYP3A4); a forward primer 5′-ATGAAAGAAAGTCGCCTCG-3′ and a reverse primer 5′-TGGTGCCTTATTGGGTAA-3′, hepatocyte nuclear factor 4α (HNF-4α); a forward primer 5′-CCACGGGCAAACA CTACGG-3′ and a reverse primer 5′-GGCAGGCTGCTGT CCTCAT-3′, tyrosine aminotransferase (TAT); a forward primer 5′-CCCCTGTGGGTCAGTGTT-3′ and a reverse primer 5′-GTGCGACATAGGATGCTTTT-3′, tryptophan 2, 3-dioxygenase (TDO2); a forward primer 5′-TACAGAGCAC TTCAGGGAG-3′ and a reverse primer 5′-CTTCGGTATCC AGTGTCG-3′, glyceraldehyde 3 phosphate dehydrogenase (GADPH); a forward primer 5′-GAAGGTGAAGGTCGGA GTC-3′ and a reverse primer 5′- GAAGATGGTGATGGG ATTTC-3′.

Quantitative RT-PCR

Total RNA was isolated by using TRIzol reagent and cDNA was synthesized in 20 μL of reaction volume containing 1 μg of total RNA and Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) in accordance with the manufacturer's instructions. Duplex real-time PCR (target gene and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) as a reference gene) in 96-well optical plates was performed using TaqMan® technology and analyzed using an ABI PRISM® PE7900 HT sequence Detection System (Perkin-Elmer Applied Biosystems, Lincoln, CA). PCR mix per well (25 μL) consisted of commercially available, premixed GAPDH TaqMan® primers/probe, TaqMan® Gene Expression Assays, inventoried primers/probe for the target gene, 0.5 μL cDNA and QuantiTect® Multiplex PCR Master Mix (Qiagen, Valencia, CA). PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. The expression level of each gene was normalized to RNA content for each sample by using GAPDH as an internal control.

Western blotting

Western blotting was performed using rabbit polyclonal antihuman AFP (Epitomics Inc., Burlingame, CA), rabbit polyclonal antihuman ALB (DakoCytomation, Glostrup, Denmark), rabbit polyclonal antihuman AAT (Lifespan Bioscience Inc., Seattle, WA), and rabbit polyclonal anti-human CYP 3A4 antibodies (Abcam, Cambridge, UK). The second antibody reaction was performed using a horseradish peroxidase-conjugated antirabbit or antimouse IgG (Cell Signaling Technology, Inc., Beverly, MA). The final detection procedure was performed using ECL Western blotting detection reagents (GE Healthcare UK Ltd., Buckinghamshire, UK).

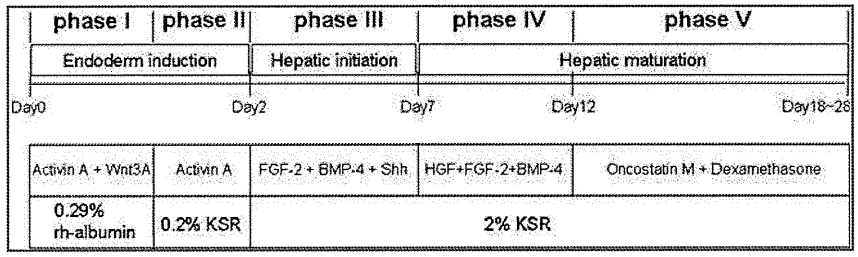


FIG. 1. Schematic presentation of feeder-free and serum-free production of functional hepatocytes from human iPS and ES cells. Undifferentiated human iPS and ES cells were induced to differentiate into hepatocytes using a five phase culture system that mimics developmental process of liver *in vivo*. Abbreviations: FGF-2: fibroblast growth factor 2, BMP-4: bone morphogenic protein 4, Shh: Sonic hedgehog, HGF: hepatocyte growth factor, KSR: Knockout™ Serum Replacement, rh-albumin: recombinant human albumin.

Immunostaining

The cells were fixed with acetone/methanol solution (1:3), permeabilized with 0.1% Triton X-100 in phosphate-buffered saline and blocked with BlockAce (DS Pharma Biomedical). The immunostaining procedure was performed with primary antibody reactions using a rabbit polyclonal antihuman AFP antibody (Epitomics), rabbit polyclonal antihuman AAT antibody (Lifespan Bioscience), rabbit polyclonal antihuman CYP3A4 (Abcam), mouse monoclonal antihuman epithelial cell adhesion molecule (EpCAM) antibody (Cell Signaling Technology), and a rabbit polyclonal antihuman ALB antibody (DakoCytomation), followed by secondary antibody reactions using Alexa Fluor® 488 chicken antimouse IgG (H+L), Alexa Fluor® 568 goat anti-rabbit IgG (H+L) or Alexa Fluor® 594 chicken antigoat IgG (H+L) (Invitrogen) antibodies.

Periodic Acid Schiff (PAS) assay for glycogen storage

Glycogen storage was measured by PAS staining a using a PAS staining kit (Muto Pure Chemicals, Tokyo, Japan) in accordance with the manufacturer's instructions.

Cellular uptake and release of Indocyanine Green (ICG)

ICG (Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO to make a stock at 5 mg/mL and then freshly diluted in culture medium to 1 mg/mL. After incubation of cells with ICG (Sigma-Aldrich) for 30 min at 37°C, the medium with ICG was discarded and washed three times with phosphate-buffered saline, and the cellular uptake of ICG was examined by microscopy. Cells were then returned to the culture medium and incubated for 6 h for the release of cellular ICG stain.

Cytochrome P450 activity assay

CYP3A4 activity was evaluated using a p450-GloTM CYP3A4 Assay kit (Promega, Madison, WI). The cells were treated with or without dexamethasone (50 μM) for 16 h for induction and were then incubated with culture medium supplemented with 50 μM CYP3A4 substrates in accordance with the manufacturer's instructions. At 4 h after treatment, 50 μL of culture medium was removed and assayed in a luminometer. CYP450 activities were expressed as relative light units (RLU/mL).

Hepatocyte toxicity assay by D-galactosamine (D-GalN)

The cells were treated with 25 mM D-GalN for 24 h at 37°C, and then the supernatant was collected. Glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), γ-glutamyl transpeptidase (γ-GTP), leucine aminopeptidase (LAP), and isozymes of lactate dehydrogenase (LDH) in the culture medium were measured sing a routine colorimetric laboratory method. In some experiments, cells were preincubated with 4 mM prostaglandin E1 (PGE1) for 4 h at 37°C before D-GalN was added.

Transmission electron microscopy

The samples were fixed using 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) for 2 h. After washing with PBS, samples were postfixed with 2% osmium tetraoxide for 1 h. Samples were dehydrated in a series of ascending ethanol concentrations and placed in propylene oxide prior to embedding in epoxy resin (Sakura Finetek Japan, Tokyo, Japan). After resin polymerization, sections of approximately 60~80 nm were cut using Ultracuts (Reichert Scientific Instruments Co., Buffalo, NY) and double stained with uranyl acetate and lead citrate. Electron micrographs were taken using a Hitachi H-7500 transmission electron microscope (Hitachi High-Technologies Corp., Tokyo, Japan).

Cell proliferation assay

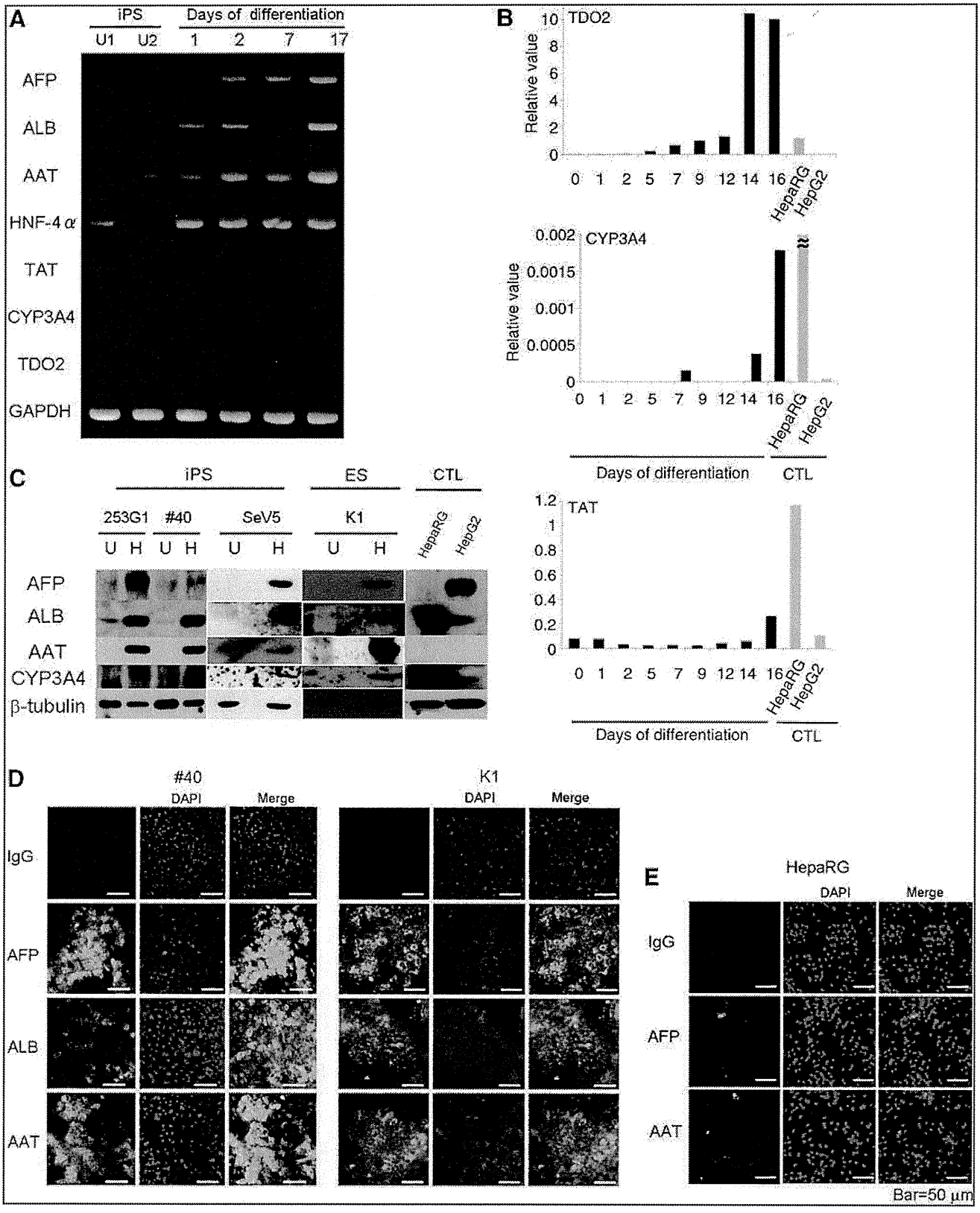
We used a Cellomics® BrdU and Ki-67 cell proliferation kit (Thermo Fisher Scientific Inc., Waltham, MA) and the assay was performed in accordance with the manufacturer's instructions. The cells were labeled with 120 μM BrdU for 20 h and then permeabilized and incubated with mouse anti-BrdU and rabbit

FIG. 2. Molecular characterization of hepatocytes induced from human iPS and ES cells. (**A**) RT-PCR analysis of hepatocyte-specific marker genes (AFP: α-fetoprotein, ALB: Albumin, AAT: α1-antitrypsin, HNF-4α: hepatocyte nuclear factor 4α, TAT: tyrosine aminotransferase, CYP3A4: cytochrome P450 3A4, TDO2: tryptophan 2, 3-dioxygenase, GADPH: glyceraldehyde-3-phosphate dehydrogenase) in undifferentiated human iPS cells (253G1) and cells derived from human iPS cells (253G1) during differentiation inducting cultures. Each lane of the RT-PCR analysis indicated undifferentiated human iPS cells (253G1) cultured on MEF (U1), undifferentiated human iPS cells cultured on Matrigel (U2), induced cells on the first (1), second (2), seventh (7), and seventeenth (17) days of differentiation. (**B**) Quantitative RT-PCR analysis of three hepatocyte-specific marker genes (TDO2, CYP3A4, TAT) in undifferentiated human iPS cells (253G1) and cells derived from human iPS cells (253G1) during differentiation induction cultures. As a control (CTL), two human hepatic cell lines were used. These two cell lines were HepaRG cells (HepaRG) and HpG2 cells (HepG2). The sign " $\approx$ " on the top of the HepaRG signal for CYP3A4 indicates that the value was beyond the vertical scale of the graph and the relative value was 1.033. (**C**) Western blot analysis of hepatocyte-specific markers (AFP: α-fetoprotein, ALB: Albumin, AAT: α1-antitrypsin, CYP3A4: cytochrome P450 3A4) in undifferentiated (U) human iPS cells (253G1, #40, and SeV5 cells) and human ES cells (K1: KhES-1 cells) and induced cells on the 29th day of differentiation (H). As a control (CTL), two human hepatic cell lines were used. These two cell lines were HepaRG cells (HepaRG) and HpG2 cells (HepG2). (D) Immunostaining of hepatocyte-specific markers of cells induced from human iPS cells (#40) and human ES cells (K1: KhES-1 cells). Human iPS and ES cells were induced to differentiate for 29 days and were stained using an isotype control antibody (IgG), a rabbit polyclonal antihuman α-fetoprotein (AFP) antibody, a rabbit polyclonal antihuman albumin antibody (ALB), or a rabbit polyclonal antihuman α-1 antitrypsin (AAT) as indicated. A total 1 μg/mL DAPI was used to stain the cell nucleus. Scale bar = 50 mm. (E) Immunostaining of hepatocyte-specific markers of HepaRG cells were performed using an isotype control antibody (IgG), a rabbit polyclonal antihuman α-fetoprotein (AFP) antibody, or a rabbit polyclonal anti-human α-1 antitrypsin (AAT) antibody as indicated. A total of 1 μg/mL DAPI was used to stain the cell nucleus. Scale bar = 50 mm.

anti-Ki-67 antibodies for 60 min at 37°C. After washing, the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG, Alexa Fluor 549-conjugated goat antirabbit IgG and DAPI, washed twice, and mounted on glass slides with ProLong® Gold antifade reagent (Invitrogen). The slides were subsequently inspected using a fluorescence microscope.

Experiments for passages and regrowth of induced hepatocytes

At the 28th day of differentiation, induced hepatocytes were collected from the original culture dishes using a StemPro® EZPassage™ Disposable Stem Cell Passaging Tool



(Invitrogen) and were recultured in new 3.5 mmϕcollagen-coated dishes (ASAHI GLASS Co., Ltd., Tokyo, Japan).

Results

Feeder-free and serum-free culture methods for hepatocyte differentiation of human iPS and ES cells

After a process of trial and error, we found that the key to success in inducing endodermal differentiation of human ES/iPS cells resided in preparing the starting materials at appropriate densities, that is, to seed the undifferentiated human ES/iPS cells so that the clumps of cells were just contacting one another. By using these appropriately seeded cells, we successfully performed a three-stage (five-phase) differentiation protocol for the induction of functional hepatocytes from human iPS and ES cells (Fig. 1).

In the initial experiment, we performed differentiation induction of human iPS cells (253G1) established from adult human dermal fibroblasts using retrovirus vectors, because this cell line is one of the standard iPS cell lines in Japan and was established without using a protooncogene c-Myc. During differentiation induction, we analyzed time courses of expression of hepatocyte-specific genes (AFP, ALB, and hepatocyte-specific metabolic enzymes). As shown in Figure 2A, transcriptional expression of hepatocytes-specific markers such as AFP, ALB, AAT, tyrosine aminotransferase (TAT), CYP3A4, and tryptophan 2, 3-dioxygenease (TDO2) were observed during differentiation, although the expression levels of TAT, CYP3A4, and TDO2 were slightly weaker than the other markers. To evaluate more quantitatively, we then performed quantitative real time PCR studies of these three markers, TAT, CYP3A4, and TDO2 and compared them with those of human hepatocyte cell lines, HepaRG and HepG2. As shown in Figure 2B, cellular expression of these three markers was detected in a time-dependent manner and the level of their expression was quantitatively equivalent to or sometimes much better than the level of human hepatocyte cell lines.

We then confirmed the expression of hepatocyte markers at the protein level. In these experiments, we also used two other human iPS cell lines, one was #40 established from fetal fibroblasts using a retrovirus vector, and the other was SeV5 established from neonatal fibroblasts using Sendai virus vector, without integration of viral vector components and transgenes. As shown in Figure 2C, several hepatocyte markers, such as AFP, ALB, AAT, and CYP3A4, were induced in all three human iPS cells. The level of induction was similar to that of human hepatocyte cell lines. In particular, AAT, which was not detected in control human hepatocyte cell lines, was prominently induced in human iPS cells. These findings were further confirmed using human ES cells (KhES-1) (Fig. 2C). Protein expression of AFP, ALB, and AAT was also confirmed by immunostaining (Fig. 2D), and overall positivity for these three markers was approximately 30–60%. In contrast, protein expression of AFP and AAT in HepaRG cells as determined by immunostaining was weak and positivity was less than 20% (Fig. 2E).

Functional evaluation of hepatocytes induced from human iPS and ES cells

To clarify whether human hepatocytes induced from human pluripotent stem cells possessed the functional capacity of mature hepatocytes, we performed three standard assays, ICG-uptake capacity, glycogen storage capacity, and CYP450 activity. As shown in Figure 3A, hepatocytes differentiated from human iPS cells (253G1, #40, and SeV5) and human ES cells (KhES-1) all showed clear uptake of ICG, and this ICG was released after 6 h. The overall level of ICG uptake-positive cells was approximately 20–30%. HepaRG and HepG2 cells showed substantial capacity (20–30% for HepaRG cells and less than 5% for HepG2 cells). Thus, hepatocytes from human iPS and ES cells have sufficiently mature functions for hepatocytes compared with the levels in control hepatocytes.

Then, we evaluated cytoplasmic glycogen accumulation in undifferentiated human iPS and ES cells and hepatocytes induced from these pluripotent stem cells, and found that almost all (more than 80%) of hepatocytes induced from human iPS and ES cells were strongly positive for PAS staining, whereas undifferentiated human iPS and ES cells were not stained (Fig. 3B). On the other hand, HepaRG cells were almost 50–60% positive, and Hep G2 cells were negative.

It has been reported that CYP3A4 plays a central role in drug metabolism and detoxification in the liver among a subfamily of CYP450s (Liu et al., 2007), and we identified the expression of this critical enzyme during our differentiation culture at both mRNA (Fig. 1) and protein (Fig. 2) levels. We then determined the functional activity of CYP3A4 using hepatocytes differentiated from human iPS and ES cells. As shown in Figure 3C, hepatocytes induced from human iPS and ES cells showed high levels of CYP3A4 activity compared with those of undifferentiated human iPS and ES cells and human hepatic cell lines, HepaRG and HepG2 cells. In addition, high CYP3A4 activity in the differentiated cells was further potentiated by transient (16 h) pharmacological induction in hepatocytes induced from human iPS cells (#40) and human ES cells (KhES-1).

Hepatocyte toxicity assay using D-galactosamine

It is essential to establish an *in vitro* hepatocyte cytotoxic assay for the evaluation of liver toxicity of various substance and/or drugs. Therefore, we performed an *in vitro* cytotoxic assay with hepatocytes induced from human pluripotent stem cells using a traditional method with D-GalN (Bao and Liu, 2010; Kuhla et al., 2009; Siendones et al., 2005). GOT, GPT, γ-GTP, LAP, and LDH isozymes released from the cells were quantified as an indicator of hepatocyte-specific cytotoxicity.

As shown in Figure 4A, D-GalN significantly and potently induced the extracellular release of GOT, GPT, and/or LDH into the culture medium, and the pattern of LDH was hepatocyte-specific (LDH4/5 dominant) in hepatocytes induced from human iPS and ES cells. These cytotoxic effects of D-GalN were significantly inhibited by PGE1 (Fig. 4B) (Siendones et al., 2005). In contrast, D-GalN only minimally induced the extracellular release of these hepatocyte-specific enzymes in undifferentiated human iPS and ES cells and human umbilical vein endothelial cells (HUVECs). Extracellular release of GOT, GPT, and LDH5 into the medium was also observed in control hepatocytes, HepaRG and HepG2 cells. Interestingly and unexpectedly, γ-GTP and LAP, which are bile duct specific enzymes, were released from "hepatocytes" induced from human iPS and ES cells

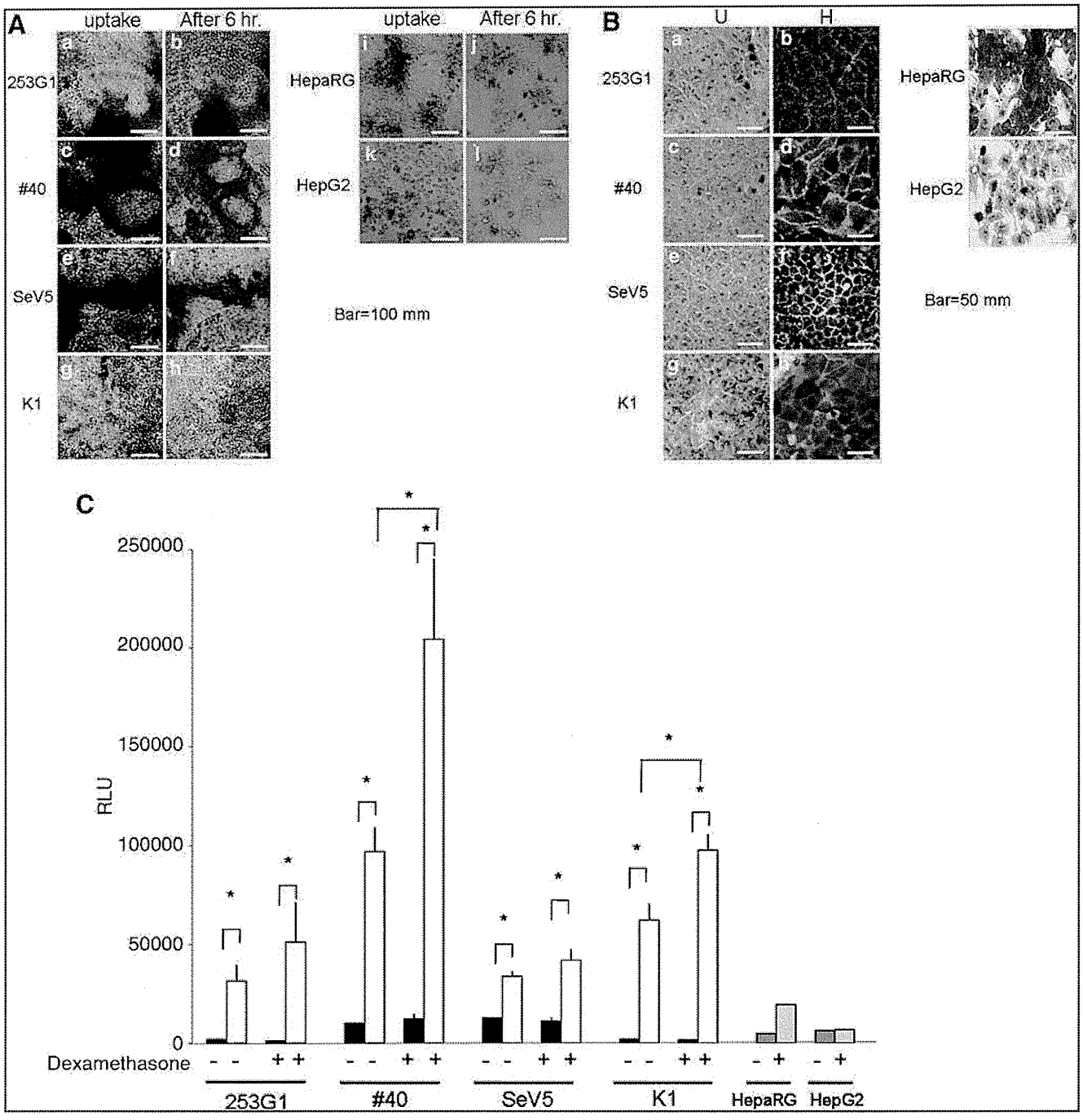


FIG. 3. Functional activity .of hepatocytes induced from human iPS and ES cells. (**A**) Indocyanine green (ICG) uptake and release. Hepatocytes induced from three human iPS cells (253G1, #40, and SeV5 cells) and ES cells (K1: KhES-1 cells) (on the 29th day of differentiation culture) and HepaRG cells (HepaRG) and HepG2 cells (Hep G2) were examined for their ability to take up ICG (left column, a, c, e, g, i, and k) and release it 6 h thereafter (right column, b, d, f, h, j, and l). Scale bar = 50 mm. (**B**) Glycogen storage ability as evaluated by Periodic Acid Schiff (PAS) staining were determined for hepatocytes induced from three human iPS cells (253G1, #40, and SeV5 cells) and ES cells (K1:KhES-1 cells) (on the 29th day of differentiation culture) and HepaRG cells (HepaRG) and HepG2 cells (Hep G2). Nuclei were counterstained with hematoxylin. Glycogen storage is indicated by pink or dark red-purple cytoplasms. Scale bar = 50 mm. (**C**) CYP3A4 activity and its induction by dexamethasone were determined using assay kit and expressed as relative light units (RLU) per milliliter of culture medium. Solid bar, open bar, and gray bar represent undifferentiated human ES/iPS cells, induced hepatocytes, and control human hepatic cell lines, respectively. Data for undifferentiated human ES/iPS cells and induced hepatocytes are presented as mean ± SD from triplicate experiments ($*p < 0.05$), and data of control human hepatic cell lines are the means of single experiment.

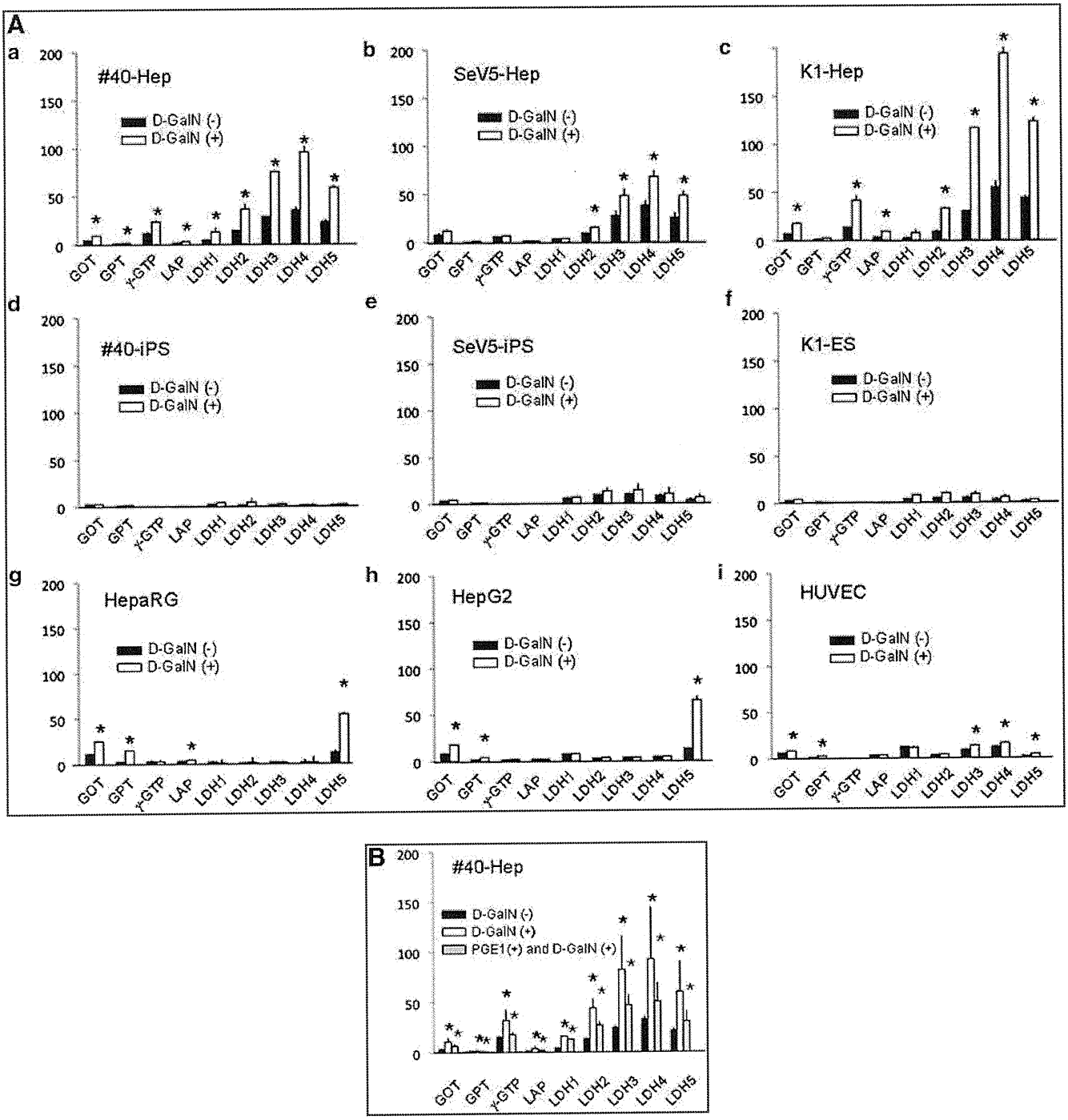


FIG. 4. D-galactosamine cytotoxic assay. (**A**) Cells were incubated with 25 mM D-galactosamine (D-GalN) for 24 h at 37°C. Liver-specific enzymes (GOT, GTP, γ-GTP, LAP, isozymes of LDH) released from the cells into the culture medium were measured using a routine colorimetric laboratory method and expressed as mean ± SD of triplicate assays. Hepatocytes induced from two human iPS cells (#40-Hep, SeV5-Hep) and ES cells (K1-Hep) (on the 29th day of differentiation culture), undifferentiated iPS/ES cells (#40-iPS, SeV5-iPS, K1-ES) and HepaRG cells (HepaRG), HepG2 cells (HepG2) and human umbilical vein endothelial cells (HUVEC) were used in this assay. $^*p < 0.05$. (**B**) The cells were preincubated with 2 mM prostaglandin E1 (PGE1) for 2 h at 37°C, and were then incubated with 25 mM D-galactosamine (D-GalN) for 24 h at 37°C. Liver-specific enzymes (GOT, GTP, γ-GTP, LAP, isozymes of LDH) released from the cells into the culture medium were measured by a colorimetric routine laboratory method and expressed as mean ± SD of triplicate assays. Hepatocyte-like cells induced from human iPS cells (#40-Hep) at the 29th days of differentiation were used in this assay. $^*p < 0.05$.

but not undifferentiated human iPS and ES cells, HUVEC, and control hepatocytes (HepaRG and HepG2 cells). These findings suggest that "hepatocytes" induced from human iPS and ES cells include not only hepatocytes themselves but also bile duct-related cells such as cholangiocytes. Thus, our culture system could be useful for cytotoxicity assays for both hepatocytes and cholangiocytes.

Electron microscopic study of hepatocytes and cholangiocytes in our culture system

To confirm that the "hepatocytes" induced from human pluripotent stem cells were really hepatocytes and explore possible coexistence of bile ducts, bile canaliculi, and cholangiocytes in the culture system, we performed detailed morphological examinations of differentiated cells using an electron microscope.

As shown in Figure 5A, hepatocytes induced from human ES cells were equipped with microvilli of intermediate length (shorter than the microvilli in the intestine) on their open space-side ["space of Disse" (perisinusoidal space) in the liver] and adjacent cells were connected via structures such as tight junctions and desmosomes. Between the cells, there was a microduct-like structure with microvilli on the lumen side, which was considered to be bile canaliculus, and this microduct-like structure was also joined by cell–cell connecting structures (tight junctions and desmosomes). Abundant glycogen α-particles were observed in the cytoplasm, and these glycogen α-particles showed a hepatocyte-specific "rosette formation." We also observed "peroxisomes," cytoplasmic structures highly specific for hepatocytes. Similar microscopic findings were observed in hepatocyte-like cells induced from human iPS cells (Fig. 5B). In addition, typical structures specific for bile ducts were also observed, that is, bile duct epithelial cells with short microvilli on the lumen side and the basement membrane on the other side were observed (Fig. 5C).

Thus, there existed at least three cell types and structures, hepatocytes, bile canaliculi (intrahepatic microbile duct) and bile duct epithelial cells, and all of these microstructures are extremely specific for the liver (Ghadially, 1997).

Possible existence of bipotential hepatoblasts with proliferating capacity

Data presented in Figures 4 and 5 together clearly indicate the presence of both hepatocytes and cholangiocytes in the present culture system. It is has been proposed that both hepatocytes and cholangiocytes are derived from their common progenitor cells called hepatoblasts (Zhao and Duncan, 2005). We then explored the presence of immature progenitors with proliferating potential.

Figure 6A shows cell morphologies observed using an inverted microscope. Two forms of cell structures and/or areas were observed: one was a bulging cell clump area, and the other was a flattened monolayer area containing binuclear cells. These two areas were analyzed using immunostaining for AFP and EpCAM, both of which are hepatoblasts markers (Schmelzer et al., 2006). As shown in Figure 6B, the bulging areas were stained positively for both markers, whereas the flattened areas were not. These findings suggested that the present culture system mainly contained two populations: one in the bulging area with proliferating immature cells with hepatoblast markers, and the other in the flattened mature hepatocyte area. The former area was also positive for proliferation marker Ki-67 (Fig. 6C), and the latter areas were also positive for ALB, a mature hepatocyte marker (Fig. 6D). In addition, we confirmed these findings in a single microscopic field; namely, the bulging area was positive for BrdU, a proliferation marker, and the flattened area was positive for AAT, a mature hepatocyte marker (Fig. 6E). Thus, the bulging area with frequent cell nuclei (stained by DAPI) was positive for proliferation markers (Ki-67 and BrdU) and hepatoblast markers (AFP and EpCAM), whereas the flattened area had fewer cell nuclei (stained by DAPI) and was negative or weakly positive for proliferating markers and positive for mature hepatocyte markers (ALB and AAT).

To further confirm the proliferative potential of "hepatocytes" from human pluripotent stem cells, we performed passages of differentiated cells into new culture dishes. Induced hepatocytes were collected from the original culture dish and recultured in new dishes. After attaching to the new dish, cells began to proliferate again (Fig. 6F, upper panel). On the second day of reculture, colonies of small-sized cells (10–20 μm vs. 20–60 μm; cells of flattened areas in Fig. 6A) appeared and expanded during reculture for up to 9 days (Fig. 6F, upper panel). These small-sized cells were positive for AFP and AAT (Fig. 6F, lower panel).

Thus our differentiation culture included not only mature hepatocytes but also their precursors with proliferation potential and began to proliferate again even after transfer to new culture dishes along with positive results for hepatocyte markers.

Discussion

In the present study, we established a feeder-free and serum-free induction system for human mature, functional hepatocytes from human pluripotent stem cells, iPS, and ES cells. We found that an initial high density culture, so that each colony of undifferentiated human ES/iPS cells was contacting other colonies, was essential in order to start stable and reproducible differentiation. In addition, we used a high concentration of Activin A and added Wint 3A to induce effective initial endoderm differentiation (Hay et al., 2008a). We also added Shh during second-step differentiation because Hedgehog has been reported to play important roles during liver development in embryos (Hirose et al., 2009) and liver regeneration after hepatectomy (Ochoa et al., 2010), despite the fact that there are no reports to use Hedgehog for hepatic differentiation of human ES/iPS cells.

There have been many reports to show *in vitro* differentiation of human ES/iPS cells toward hepatocytes, although most of the studies utilizes animal-derived cells and/or materials during at least one step of differentiation cultures (Agarwal et al., 2008; Cai et al., 2007; Chiao et al., 2008; Duan et al., 2007, 2010; Hay et al., 2008a, 2008b; Inamura et al., 2011; Ishii et al., 2008; Liu et al., 2010; Mfopou et al., 2010; Sasaki et al., 2009; Si-Tayeb et al., 2010; Song et al., 2009; Sullivan et al., 2010; Touboul et al., 2010; Zhao et al., 2009), such as the presence of MEF or MEF-conditioned medium at the initial step of the culture, the presence of mouse feeder cells for differentiation induction, or fetal bovine serum during the certain periods of differentiation culture. In

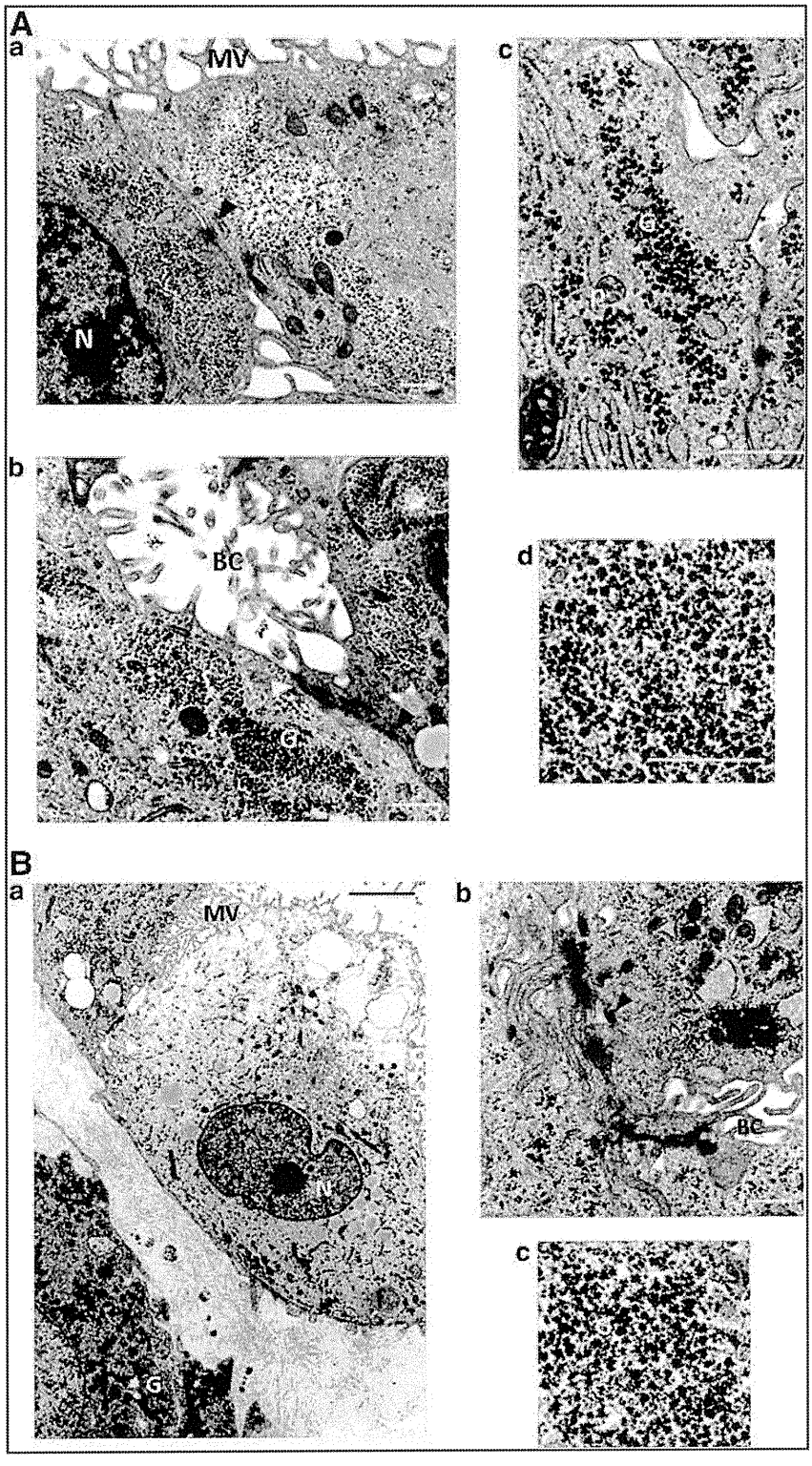


FIG. 5. Transmission electron micrographs of hepatocyte and cholangiocytes induced from human ES and iPS cells. (**A**) Hepatocytes derived from human ES cells (KhES-1 cells). (a) apical surface microvilli (MV), tight-junction (white arrowhead), desmosome (black arrowhead), and nucleus (N). (b) bile canaliculus (BC), glycogen granule (G), tight-junction (white arrowhead) and desmosome (black arrowhead). (c) glycogen granule (G) and peroxisome (p). (d) rosette formation of glycogen granules. (**B**) Hepatocytes derived from human iPS cells (SeV5) (a) apical surface microvilli (MV), glycogen granule (G), and nucleus (N). (b) bile canaliculus (BC), tight-junction (white arrowhead) and desmosome (black arrowhead). (c) rosette formation of glycogen granules. (**C**) Cholangiocytes derived from human iPS cells ((a) #40 cells and (b) SeV5 cells). Bile duct lumen (BD) side with short microvilli (white arrowhead) and outer side with basement membrane (black arrowhead). N: nucleus. White scale bar = 2 μm, and black scale bar = 10 μm.